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Inhibition of Lipopolysaccharide-Stimulated Chronic Obstructive Pulmonary Disease Macrophage Inflammatory Gene Expression by Dexamethasone and the p38 Mitogen-Activated Protein Kinase Inhibitor *N*-cyano-*N′*-(2-{[8-(2,6-difluorophenyl)-4-(4-fluoro-2-methylphenyl)-7-oxo-7,8-dihydropyrido[2,3-*d*]pyrimidin-2-yl]amino}ethyl)guanidine (SB706504)[S]

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ABSTRACT

p38 mitogen-activated protein kinase (MAPK) signaling is known to be increased in chronic obstructive pulmonary disease (COPD) macrophages. We have studied the effects of the p38 MAPK inhibitor *N*-cyano-*N′*-(2-{[8-(2,6-difluorophenyl)-4-(4-fluoro-2-methylphenyl)-7-oxo-7,8-dihydropyrido[2,3-*d*]-pyrimidin-2-yl]amino}ethyl)guanidine (SB706504) and dexamethasone on COPD macrophage inflammatory gene expression and protein secretion. We also studied the effects of combined SB706504 and dexamethasone treatment. Lipopolysaccharide (LPS)-stimulated monocyte derived macrophages (MDMs) and alveolar macrophages (AMs) were cultured with dexamethasone and/or SB706504. MDMs were used for gene array and protein studies, whereas tumor necrosis factor (TNF) α protein production was measured from AMs. SB706504 caused transcriptional inhibition of a range of cytokines and chemokines in COPD MDMs. The use of SB706504 combined with dexamethasone caused greater suppression of gene expression (−8.90) compared with SB706504 alone (−2.04) or dexamethasone (−3.39). Twenty-three genes were insensitive to the effects of both drugs, including interleukin (IL)-1β, IL-18, and chemokine (CC motif) ligand (CCL) 5. In addition, the chromosome 4 chemokine cluster members, CXCL1, CXCL2, CXCL3, and CXCL8, were all glucocorticoid-resistant. SB706504 significantly inhibited LPS-stimulated TNFα production from COPD and smoker AMs, with near-maximal suppression caused by combination treatment with dexamethasone. We conclude that SB706504 targets a subset of inflammatory macrophage genes and when used with dexamethasone causes effective suppression of these genes. SB706504 and dexamethasone had no effect on the transcription of a subset of LPS-regulated genes, including IL-1β, IL-18, and CCL5, which are all known to be involved in the pathogenesis of COPD.

Chronic obstructive pulmonary disease (COPD) is characterized by progressive airflow obstruction and airway inflammation (Barnes, 2003). Alveolar macrophages (AMs) are believed to play a central role in disease pathogenesis by secreting proinflammatory cytokines and chemokines (Barnes, 2003). Glucocorticoids (GCs) are the most widely used

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ABBREVIATIONS: COPD, chronic obstructive pulmonary disease; AM, alveolar macrophage; GC, glucocorticoid; GR, glucocorticoid receptor; NF, nuclear factor; TLR, Toll-like receptor; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; TNF, tumor necrosis factor; IL, interleukin; GM-CSF, granulocyte macrophage colony-stimulating factor; MDM, monocyte-derived macrophage; PCR, polymerase chain reaction; UP, ultrapure; DMSO, dimethyl sulfoxide; qPCR, quantitative PCR; ELISA, enzyme-linked immunosorbent assay; IPA, ingenuity pathways analysis; FEV, forced expiratory volume; FVC, forced vital capacity; CCL, chemokine (CC motif) ligand; SB706504, *N*-cyano-*N′*-(2-{[8-(2,6-difluorophenyl)-4-(4-fluoro-2-methylphenyl)-7-oxo-7,8-dihydropyrido[2,3-*d*]pyrimidin-2-yl]amino}ethyl)guanidine; SB239063, *trans*-1-(4-hydroxycyclohexyl)-4-(4-fluorophenyl)-5-(2-methoxypyridimidin-4-yl)imidazole; SD-282, indole-5-carboxamide (ATP-competitive inhibitor of p38 kinase).

anti-inflammatory therapy in COPD. GC suppress inflammatory gene transcription by forming a complex with the glucocorticoid receptor (GR) that inhibits the function of transcription factors such as nuclear factor (NF)-κB, a process known as transrepression (Glass and Ogawa, 2006). However, the clinical benefits of GC in COPD patients are modest (Soriano et al., 2007), and the suppression of cytokine production from COPD AMs is reported to be GC-resistant (Culpitt et al., 2003; Cosio et al., 2004). Alternative anti-inflammatory therapies are needed in COPD (Barnes, 2006).

Extracellular stimuli such as the TOLL-like receptor (TLR) 4 ligand lipopolysaccharide (LPS) and cytokines activate p38 mitogen-activated protein kinase (MAPK) intracellular signaling. This signaling pathway up-regulates the production of proinflammatory cytokines and chemokines (Zarubin and Han, 2005) through the activation of transcription factors such as NFκB and activating transcription factor 2 or alterations in chromatin structure to allow NFκB binding to the promoter regions of inflammatory genes (Saccani et al., 2002). p38 MAPK may also act post-transcriptionally through mRNA stabilization (Winzen et al., 1999) or at the level of protein translation (Newton and Holden, 2003; Brook et al., 2006). Activated p38 MAPK expression is increased in pulmonary macrophages from COPD patients (Renda et al., 2008), implicating this signaling pathway in the pathophysiology of COPD. Therefore, p38 MAPK inhibitors are in clinical development for the treatment of COPD (Barnes, 2006). In THP-1 cells and healthy human AMs, the p38 MAPK inhibitors SB203580 and 2,1(1,3-dihydroxyprop-2-yl)-4-(4-fluorophenyl)-5-(2-phenoxypyrimidin-4-yl)imidazole) inhibited LPS-stimulated cytokine protein levels but with little or no effect on cytokine mRNA levels (Birrell et al., 2006). This suggests that these p38 MAPK inhibitors act at the level of protein translation rather than gene transcription in macrophages.

GCs inhibit p38 MAPK activity by increasing the gene expression of MAPK phosphatase-1 (Lasa et al., 2002). In addition, p38 MAPK inhibitors may enhance the effects of GC (Irusen et al., 2002) by altering the phosphorylation of the GR (Szatmáry et al., 2004). These potentially synergistic interactions provide a good rationale to use these two classes of drug together to maximize anti-inflammatory effects.

In AMs from controls and patients with emphysema, the p38 MAPK inhibitors SB239063 and SD-282 significantly inhibited LPS-induced tumor necrosis factor (TNF)-α protein levels but had little effect on interleukin (IL)-8 (also known as CXCL8) and granulocyte macrophage colony-stimulating factor (GM-CSF), indicating that the effects of p38 MAPK inhibitors vary between inflammatory genes (Smith et al., 2006). Likewise, ligand-activated GR does not target all inflammatory genes but represses the activity of the subset of inflammatory genes that have GR-dependent transcriptional activation (Glass and Ogawa, 2006). This phenomenon is cell type specific and is dependent on the type of stimulus used (Ogawa et al., 2005). It would be of importance to evaluate and compare the sensitivity with GC and p38 MAPK inhibitors of inflammatory genes in COPD macrophages.

The p38 MAPK inhibitor *N*-cyano-*N*′-(2-{[8-(2,6-difluorophenyl)-4-(4-fluoro-2-methylphenyl)-7-oxo-7,8-dihydropyrido-[2,3-*d*]pyrimidin-2-yl]amino}ethyl)guanidine, also known as PCG, has high specificity for the α and β p38 MAPK isoforms and low activity against other kinases (Tudhope et al., 2008). This novel MAPK inhibitor has been shown to reduce TNFα, GM-CSF, and IL-6 production from LPS-stimulated COPD macrophages, with less effect on IL-8 production, supporting previous data that some inflammatory cytokines are less sensitive to p38 MAPK inhibitors (Smith et al., 2006). We wanted to extend this observation further to profile a range of inflammatory genes to identify p38 MAPK-sensitive and -insensitive genes. Furthermore, we were interested in understanding the effects of this drug at the levels of gene transcription and when used with a glucocorticoid.

This article reports the effects of the GC dexamethasone and SB706504 on LPS activation of inflammatory genes in macrophages from COPD patients. We used monocyte-derived macrophages (MDMs) from COPD patients to perform gene array studies with the following aims: 1) to determine whether SB706504 suppressed the transcription of inflammatory genes and 2) to identify LPS-activated inflammatory genes that were insensitive to SB706504 or dexamethasone. We also studied the effects of combined treatment with SB706504 and dexamethasone. Quantitative PCR and protein analysis were performed to confirm gene array findings. Finally, we present experiments using LPS-stimulated AMs from COPD patients and a control group of healthy smokers, evaluating the effects of combined treatment with SB706504 and dexamethasone.

Materials and Methods

Patients. Six subjects diagnosed with COPD according to current guidelines (GOLD, 2008) provided blood samples for MDM cell culture and transcriptomic analysis. These patients all had oxygen saturations > 93% on air and were not using supplemental oxygen therapy. AMs for cell culture using the TLR2 and 4 agonist LPS and the specific TLR4 agonist ultrapure (UP)-LPS (Wakelin et al., 2006) were obtained from 11 patients diagnosed with COPD and 16 smokers with normal lung function undergoing surgical resection for suspected or confirmed lung cancer. Former smokers were defined on the basis of stopping smoking for at least 1 year. Demographics for all patients used in this study are provided in Tables 1 and 2 . The local research ethics committee approved this study, and written informed consent for each patient was obtained.

MDM Culture

Peripheral blood mononuclear cells were isolated from whole blood as described previously (Stengel et al., 1998; Kent et al., 2008). These cultures have been shown previously to consist of entirely CD68+ macrophages (Stengel et al., 1998), and we obtained similar data in MDMs from three subjects using flow cytometry (data not shown). Media were removed from MDMs before the addition of drugs (1 ml/well), 100 nM dexamethasone (Sigma-Aldrich, St. Louis, MO), and/or 3 μM SB706504 (gift from GlaxoSmithKline) previously stored in DMSO. This concentration was chosen based on previous

TABLE 1
Demographics of patients used within MDM study
Results are expressed as mean (S.D.).

	COPD (n = 6)
Sex	Male
Current/ex-smoker	1/5
Age	66.8 (4.3)
FEV_1	1.0 (0.5)
FEV_1 percentage predicted	32.7 (15.7)
FEV_1/FVC ratio	40.4 (13.6)
Pack year history	49.7 (20–100)[a]
ICS users	4

ICS, inhaled corticosteroid.
[a] Range.

TABLE 2
Demographics of patients used within AM study
Results expressed as mean (SD).

	COPD	Smoker
Sex (female/male)	3/8	7/9
Age	69.82 (7.1)	63.25 (11.0)
FEV_1	1.63 (0.4)	2.47 (0.7)
FEV_1 % predicted	62.55 (15.7)	91.20 (15.4)
FEV_1/FVC ratio	56.11 (10.1)	74.80 (7.7)
Pack year history	50.14 (17.5–100)[a]	42.70 (10–100)[a]
Current/ex-smoker	6/5	12/4
ICS users	9	N.A.

N.A., not applicable; ICS, inhaled corticosteroid.
[a] Range.

data (Tudhope et al., 2008) showing that the maximal effect of this compound on p38 activation was reached at 10 μM. Cells were preincubated for 30 min with drugs before incubation for 6 h with control or LPS (1 μg/ml, *Salmonella abortus equi*; Sigma-Aldrich) media. Control media were spiked with DMSO at a ratio of 1:1265 μl to simulate the highest concentration applied with the inhibitors. Cells were maintained at 37°C and 5% CO_2.

AM Culture

Resected lung tissue was obtained from areas distant to the tumor and perfused with sterile 0.15 M NaCl to isolate macrophages. Retrieved fluid was centrifuged (10 min, 400*g*, room temperature) and resuspended in RPMI 1640 media. The cell suspension was floated over a Ficoll gradient, centrifuged (30 min, 400*g* at 4°C), and cells were counted by trypan blue exclusion. Cells were then centrifuged, and the pellet was resuspended at a concentration of 1×10^6 macrophages/ml in RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen, Paisley, UK), 1% penicillin/streptomycin (Sigma Chemical, Poole, Dorset, UK), and 1% L-glutamine (Invitrogen). In 96-well plates, 0.1×10^6 cells/well were seeded and left at 37°C and 5% CO_2 for a minimum of 18 h to allow AMs to adhere to the plate. Nonadherent cells were removed by washing with supplemented RPMI 1640 medium before use. Immunohistochemistry experiments showed that these cells had >90% CD68 expression (for details of method, see supplemental data). Media were removed before the addition of 200 μl of supplemented RPMI 1640 media with 100 nM dexamethasone and/or 3 μM SB706504. AMs were preincubated for 30 min with drugs before incubation for 24 h with control, LPS (1 μg/ml, *Salmonella abortus equi*; Sigma-Aldrich), or UP-LPS (1 μg/ml; InvivoGen, San Diego, CA) media. Control media were spiked with DMSO at a ratio of 1:1265 μl to simulate the highest concentration applied with the drugs. AMs were maintained at 37°C and 5% CO_2.

Transcriptomic Analysis

RNA Isolation and Microarray Analysis. Cells were lysed in TRIzol (Invitrogen), and protocols for RNA isolation, quantification and quality assessment, and Affymetrix microarray (Affymetrix, Santa Clara, CA) analysis were as described previously (Kent et al., 2008).

Quantitative PCR. qPCR was carried out in duplicate using 250-ng aliquots of total RNA. The RNA was converted into cDNA using the ABI High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Six nanograms of cDNA was reacted with ABI 2× TaqMan PCR mix in a 10-μl reaction including 0.4 μl of the appropriate forward and reverse primers (10 μM) and 0.2 μl of the TaqMan probe (5 μM) (Genosys, Cambridge, UK). The sequences of primers and TaqMan probes are provided in Supplemental Tables 1 and 2. Thermal cycling was carried out on an ABI 7900 Sequence Detection System (Applied Biosystems) with the following profile: 50°C for 10 min, 95°C for 10 min, 92°C for 15 s, and 60°C for 1 min for 45 cycles. The housekeeping genes, β-actin, glyceraldehyde-3-phosphate dehydrogenase, and cyclophilin, were used to assess any loading affects. Quantitation was relative to a standard curve (genomic DNA) according to manufacturer's instructions. Housekeeping gene expression was found to be consistent across all samples (data not shown).

Supernatant Protein Analysis

TNFα and IL-8 Enzyme-Linked Immunosorbent Assay. Sandwich enzyme-linked immunosorbent assays (ELISAs) for TNFα and IL-8 were carried out on cell culture supernatants according to

TABLE 3
Dexamethasone- and SB706504-responsive LPS-induced inflammatory mediator probe sets in COPD MDMs

Name	Affymetrix Identification	Description	Fold Change			
			LPS[a]	Dexamethasone[b]	SB706504[b]	Dexamethasone and SB706504[b]
CCL7	208075_s_at	Chemokine (C-C motif) ligand 7	2.04	−4.58	−2.52	−8.17
TNFSF9	206907_at	TNF (ligand) superfamily, member 9	5.81	−2.67	−2.21	−3.73
CSF2	210229_s_at	Colony stimulating factor 2 (granulocyte-macrophage)	10.13	−7.04	−4.40	−21.28
LIF	205266_at	Leukemia inhibitory factor	4.92	−4.39	N.S.	−7.47
CCL2	216598_s_at	Chemokine (C-C motif) ligand 2	3.61	−7.26	N.S.	−19.27
TNFSF15	221085_at	TNF (ligand) superfamily, member 15	7.74	−4.59	−2.45	−7.26
TNFSF4	207426_s_at	TNF (ligand) superfamily, member 4	2.59	−2.19	N.S.	−2.38
IL12B	207901_at	Interleukin 12B	49.32	−7.09	−2.78	−8.72
TNF	207113_s_at	TNF (TNF superfamily, member 2)	15.30	−4.33	−2.93	−14.09
IFNG	210354_at	Interferon, γ	4.47	−5.04	−3.01	−10.87
CXCL10	204533_at	Chemokine (C-X-C motif) ligand 10	15.20	−4.12	−3.77	−13.70
IL27	1552995_at	Interleukin 27	6.19	−2.80	−2.22	−5.08
CLCF1	219500_at	Cardiotrophin-like cytokine factor 1	3.82	−2.13	−2.35	−4.60
OSM	230170_at	Oncostatin M	2.22	−2.33	N.S.	−2.65
CCL8	214038_at	Chemokine (C-C motif) ligand 8	14.51	−4.27	−3.43	−20.25
IL6	205207_at	Interleukin 6 (interferon, β 2)	20.86	−3.71	−3.39	−13.22
IL1A	210118_s_at	Interleukin 1, α	26.06	−4.94	−3.89	−16.84
CXCL9	203915_at	Chemokine (C-X-C motif) ligand 9	4.58	−2.26	−2.10	−6.53
IL10	207433_at	Interleukin 10	3.55	−2.28	−2.07	−3.40
CXCL2	209774_x_at	Chemokine (C-X-C motif) ligand 2	14.43	N.S.	−3.32	−5.21
CXCL3	207850_at	Chemokine (C-X-C motif) ligand 3	8.75	N.S.	N.S.	−3.78
CXCL1	204470_at	Chemokine (C-X-C motif) ligand 1	10.12	N.S.	N.S.	−3.37
IL8	211506_s_at	Interleukin 8	7.58	N.S.	N.S.	−2.90

$p < 0.01$ unless fold change stated as N.S.
[a] Fold change is relative to unstimulated MDMs.
[b] Fold change is LPS-stimulated MDMs plus drug relative to LPS only-stimulated MDMs.

TABLE 4
Dexamethasone- and SB706504-insensitive LPS-induced inflammatory mediator probe sets in COPD MDMs

Name	Affymetrix Identification	Description	LPS[a]
EBI3	219424_at	Epstein-Barr virus-induced gene 3	50.17
IL1B	205067_at	Interleukin 1, β	16.80
CCL1	207533_at	Chemokine (C-C motif) ligand 1	15.91
CCL15	210390_s_at	Chemokine (C-C motif) ligand 15	11.26
CCL4	204103_at	Chemokine (C-C motif) ligand 4	5.71
CCL3	205114_s_at	Chemokine (C-C motif) ligand 3	3.57
CCL20	205476_at	Chemokine (C-C motif) ligand 20	49.23
CSF3	207442_at	Colony stimulating factor 3 (granulocyte)	36.55
CXCL11	210163_at	Chemokine (C-X-C motif) ligand 11	20.86
TNFSF10	214329_x_at	TNF (ligand) superfamily, member 10	13.68
PBEF1	243296_at	Pre-B-cell colony-enhancing factor 1	5.92
IL15	205992_s_at	Interleukin 15	4.47
CCL23	210549_s_at	Chemokine (C-C motif) ligand 23	4.16
CCL5	204655_at	Chemokine (C-C motif) ligand 5	3.67
IL18	206295_at	Interleukin 18 (interferon-γ -inducing factor)	3.63
CXCL13	205242_at	Chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant)	3.57
TNFSF13B	223502_s_at	TNF (ligand) superfamily, member 13b	3.13
IL1F9	220322_at	Interleukin 1 family, member 9	2.86
IL1RN	216244_at	Interleukin 1 receptor antagonist	2.81
CD70	206508_at	CD70 molecule	2.73
CCL18	32128_at	Chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	2.22
XCL2	206366_x_at	Chemokine (C motif) ligand 2	2.18
CXCL5	214974_x_at	Chemokine (C-X-C motif) ligand 5	2.07

[a] Fold change is relative to unstimulated MDMs ($p < 0.01$).

the manufacturer's instructions (R&D Systems Europe Ltd, Abingdon, Oxfordshire, UK). Supernatants were diluted with RPMI 1640 as appropriate for ELISA analysis.

Luminex Analysis. Methodology for microsphere-antibody coupling and Luminex supernatant protein analysis for IL-6, IL-10, interferon γ-inducible protein 10, and TNFα is provided in the supplemental data. This procedure gave a range of 2.4 to 10,000 pg/ml; thus, samples were diluted as appropriate in RPMI 1640. All data points that were less than the lower limit of detection were given a value of half the lower limit (Laan et al., 2002).

Statistical Analysis

Gene array data quality was assessed for homogeneity of quality control metrics by principal component analysis using SIMCA-P+ software (Umetrics, Windsor, UK). Global analysis of gene expression was initially processed by normalizing probe intensity data using Rosetta Resolver (Rosetta Inpharmatics LLC, Seattle, WA) (Weng et al., 2006) before loading into SIMCA-P+ for visual assessment of key trends by gene expression principal component analysis. Further analysis was performed on the intensities by mixed-model analysis of variance using SAS Software (SAS Institute, Cary, NC). The experimental condition was included as a fixed factor, and donor was included as a random factor. A probe set was retained for further analysis if $p < 0.05$ in at least three of the patients. The Dunnett's post hoc test was used to compare results to control. All probe sets with $p \leq 0.01$ and fold change of 2 were deemed significant. Probe set gene names were attained from http://www.affymetrix.com.

Cytokine and chemokine genes were identified using Ingenuity Pathways Analysis (IPA; Ingenuity Systems, http://www.ingenuity-.com). IPA was also used to identify networks of interacting genes (Juric et al., 2007; Li et al., 2007; Savli et al., 2008). The program also includes genes or complexes that are not in the uploaded gene list but are also highly connected to the uploaded genes. Each gene or complex is classified as a node, and interacting nodes are defined by either direct relationships, which require direct physical contact, or indirect relationships, whereby direct physical contact is not required. The National Center for Biotechnology Information database was also used to manually search for the functions of genes and Online Mendelian Inheritance in Man database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim) used to assess gene chromosomal loci. Heatmaps were generated using Heatmap Builder Version 1.0 (Dr. Euan Ashley, Stanford University, Palo Alto, CA).

RNA and protein data were analyzed using a nonparametric analysis of variance (Friedman's test with Dunn's post hoc analysis),

TABLE 5
Dexamethasone- and SB706504-responsive inflammatory mediator probe sets in COPD MDMs whereby LPS has no effect or suppresses expression

Name	Affymetrix Identification	Description	Fold Change			
			LPS[a]	Dexamethasone[b]	SB706504[b]	Dexamethasone and SB706504[b]
IL1RN	216243_s_at	Interleukin 1 receptor antagonist	N.S.	−5.95	−3.13	−8.07
TNFSF10	202687_s_at	TNF (ligand) superfamily, member 10	N.S.	−3.99	−2.62	−9.52
CXCL11	211122_s_at	Chemokine (C-X-C motif) ligand 11	N.S.	−3.76	N.S.	−11.95
CSF1	211839_s_at	Colony-stimulating factor 1 (macrophage)	N.S.	−4.22	N.S.	−4.16
TNFSF7	206508_at	TNF (ligand) superfamily, member 7	N.S.	−2.04	N.S.	−3.13
PBEF1	1555167_s_at	Pre-B-cell colony-enhancing factor 1	N.S.	N.S.	−2.83	−3.71
PF4	206390_x_at	Platelet factor 4 [chemokine (C-X-C motif) ligand 4]	N.S.	N.S.	N.S.	2.08
LTB	207339_s_at	Lymphotoxin β (TNF superfamily, member 3)	N.S.	N.S.	N.S.	−2.52
XCL2	214567_s_at	Chemokine (C motif) ligand 2	N.S.	N.S.	N.S.	−3.75
FASLG	210865_at	Fas ligand (TNF superfamily, member 6)	N.S.	N.S.	N.S.	−2.93
TNFSF12	205611_at	TNF (ligand) superfamily, member 12	−2.72	2.45	3.09	2.39
TNFSF14	207907_at	TNF (ligand) superfamily, member 14	−5.00	N.S.	2.65	N.S.

$p < 0.01$ unless fold change stated as N.S.
[a] Fold change is relative to unstimulated MDMs.
[b] Fold change is LPS-stimulated MDMs plus drug relative to LPS only-stimulated MDMs.

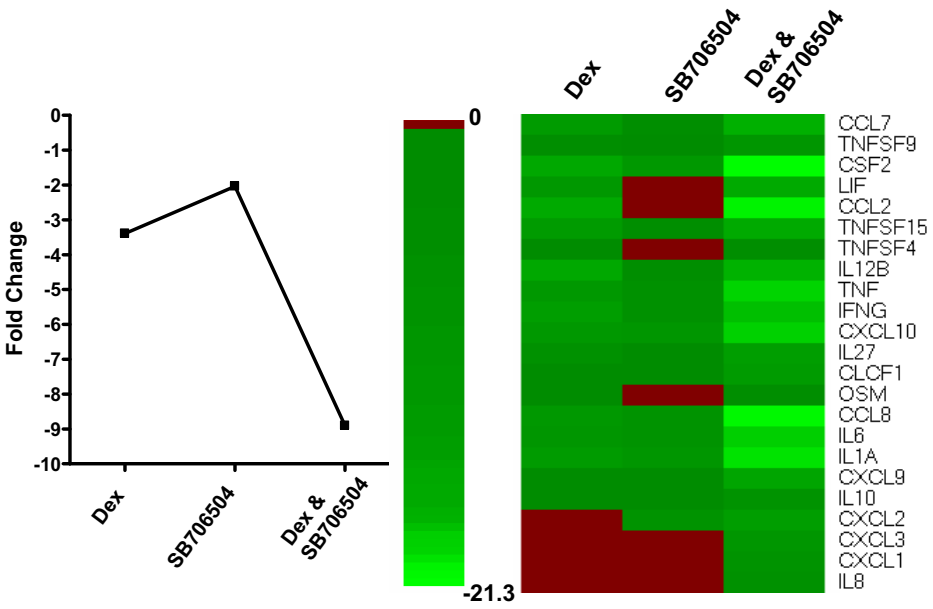

Fig. 1. Heatmap comparison of inflammatory mediator mRNA levels sensitive to dexamethasone, SB706504, or cotreatment in LPS-stimulated COPD MDMs ($n = 6$). mRNA levels were measured by microarray. Significantly regulated (minimum of a 2-fold change, $p < 0.01$) inflammatory genes were loaded into Heatmap Builder Version 1.0, and the line graph shows the mean fold change for each condition.

followed by Wilcoxon matched-pairs signed-ranks one-tailed test to test for differences between treatments. Differences between groups (COPD patients and smokers) were assessed using Mann-Whitney test. Spearman's Rank Correlation Coefficient was used to assess the relationship between array and qPCR data. Tests were performed using GraphPad InStat Software (GraphPad Software Inc., San Diego, CA).

Results

MDM Cell Culture

Gene Expression Microarrays. Fifty-eight inflammatory mediators that were significantly regulated in at least one experimental condition (LPS alone or treatment with dexamethasone and/or SB706504) with a fold change > 2 and a $p < 0.01$ were identified using IPA and manual gene cross-referencing (Tables 3–5). Twenty-three genes were significantly up-regulated by LPS and inhibited by dexamethasone, SB706504, and/or combined treatment (Table 3). Four of these genes, CXCL1, 2, and 3 and IL-8, were resistant to dexamethasone treatment but sensitive to the combined therapy (Table 3). Online Mendelian Inheritance in Man database searches for chromosomal loci also found these genes to occupy the same locus, 4q12-q13. The heatmap in Fig. 1 shows that combined treatment with dexamethasone and SB706504 caused the greatest degree of inhibition of these 23 genes (−8.90), which was more than the effect of

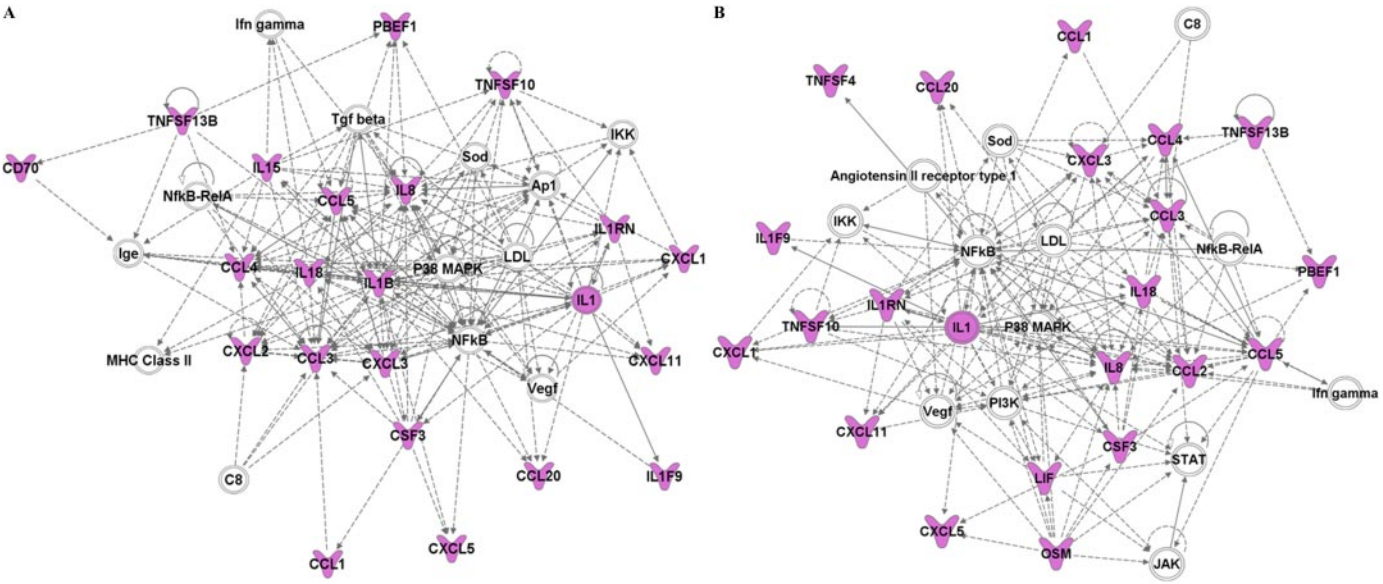

Fig. 2. Gene network generated through the use of Ingenuity Pathways Analysis (Ingenuity Systems, http://www.ingenuity.com) and derived from dexamethasone (A)- and SB706504 (B)-insensitive inflammatory mediator genes in LPS-stimulated COPD MDMs. Each gene or complex is classified as a node; V-shaped nodes define inflammatory mediators, circular nodes define complexes or groups of molecules. Groups/complexes shown here are defined as follows; IL1 (IL18, IL1β, IL1F5/6/7/8/10, and IL1RN), p38 MAPK (p38 MAPKα/β/δ/γ), NFκB (NFκB, NFκB-RelA, Rel/RelA/RelB), NFκB-RelA (NFκB plus RelA or p50/52 plus p65), and AP-1 (Jun, Fos). Nodes shaded in purple were from the original uploaded data set, and uncolored nodes were incorporated into the network by IPA because of the high levels of connectivity between the molecules. Interacting nodes are defined by either direct, which requires direct physical contact (closed arrows), or indirect, whereby direct physical contact is not required, relationships (dashed arrows). Arrow directionality gives the direction of the interaction, i.e., A "acts on" B.

dexamethasone alone (−3.39) or SB706504 alone (−2.04) when added together.

An additional 23 genes were induced by LPS but were insensitive to any of the drug treatments (Table 4). Ten genes were not induced by LPS but were sensitive to dexamethasone and/or SB706504, and a further two genes were found to be suppressed by LPS treatment and induced by dexamethasone and/or SB706504 (Table 5).

IPA analysis was applied to the 27 dexamethasone-insensitive genes to create a network of interrelated genes based on a score-centric approach (Fig. 2A). Among the most highly connected genes were IL-8, IL-1β, IL-18, and CCL5. The signaling pathways that were connected to GC-insensitive genes were p38 MAPK, NFκB, and AP-1. A similar approach was used for the 30 SB706504-insensitive genes (Fig. 2B); IL-8, IL-1β, and IL-18 were again very highly connected to other drug-insensitive genes, whereas the signaling pathways involved were p38 MAPK, as expected, NFκB, and, to a lesser extent, PI3 kinase and Janus tyrosine kinase/signal transducer and activator of transcription signaling.

qPCR. In LPS-stimulated MDMs, treatment with dexamethasone or SB706504 alone significantly reduced IL-1β, IL-6, GM-CSF, TNFα, and IL-8 mRNA levels (Fig. 3). Greater inhibition with dexamethasone compared with SB706504 was observed for IL-1β, GM-CSF, and TNFα mRNA levels. Cotreatment with dexamethasone and SB706504 caused significant further reductions in mRNA levels compared with treatment with dexamethasone or SB706504 alone for all five genes. The percentage reduction in expression levels of IL-1β, IL-6, GM-CSF, TNFα, and IL-8 mRNA caused by dexamethasone are shown in Table 6. This confirms the microarray findings that IL-8 and IL-1β were the most GC-insensitive genes. The correlation between qPCR and array changes was extremely close and statistically significant ($r = 0.91$, $p < 0.0001$).

Supernatant Proteins. In LPS-stimulated MDMs, treatment with dexamethasone or SB706504 alone significantly reduced IL-6, IL-10, interferon γ-inducible protein 10, and TNFα protein levels measured by Luminex (Fig. 4). ELISA measurements of IL-8 were numerically reduced by both of these drugs, but for SB706504, this difference did not reach statistical significance (Fig. 5). SB706504 had a greater inhibitory effect on IL-10 production compared with dexamethasone, although there were no differences for the other cytokines. Cotreatment with dexamethasone and SB706504 caused significant further reductions in the protein levels of

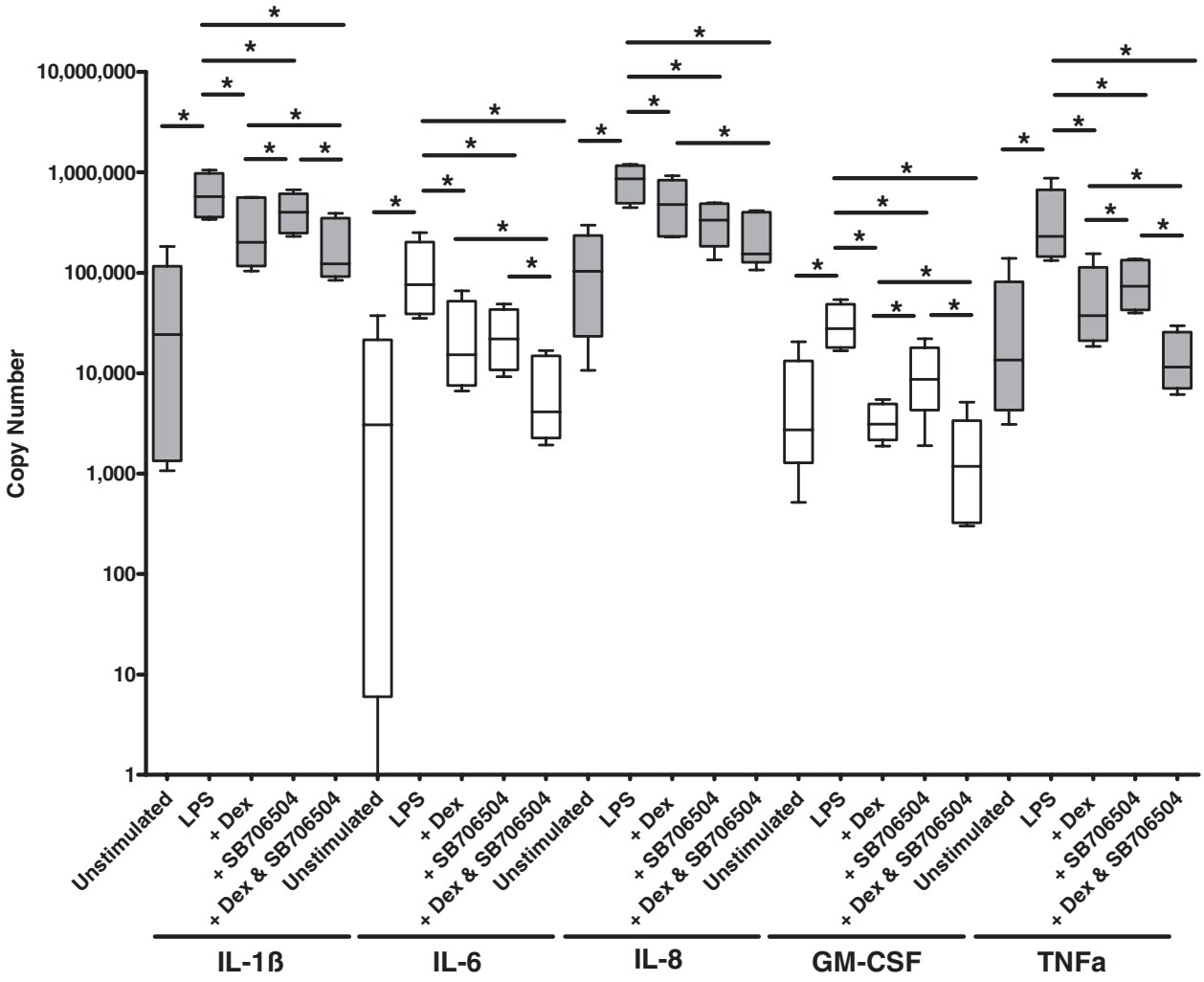

Fig. 3. Comparison of dexamethasone, SB706504, and cotreatment effects on cytokine mRNA levels measured by TaqMan qPCR in LPS-stimulated COPD MDMs ($n = 6$). Data are presented as copy number medians with interquartile range and range. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

TABLE 6
Inflammatory mediator mRNA inhibition by dexamethasone and SB706504 in LPS stimulated-COPD MDMs measured by qPCR
Fold changes are LPS-stimulated samples with drug relative to LPS only-stimulated samples, and all values have $p < 0.05$.

Gene Name	Dexamethasone		SB706504		Dexamethasone and SB706504	
	Fold Change	Median % Inhibition	Fold Change	Median % Inhibition	Fold Change	Median % Inhibition
IL-1β	−2.65	58.17	−1.51	32.49	−4.07	69.75
IL-6	−4.75	75.57	−3.99	74.76	−17.91	93.48
IL-8	−1.89	36.02	−2.59	58.63	−4.63	68.61
GM-CSF	−9.85	88.03	−4.11	66.91	−46.37	95.27
TNFα	−6.46	84.77	−3.88	71.47	−23.86	95.16

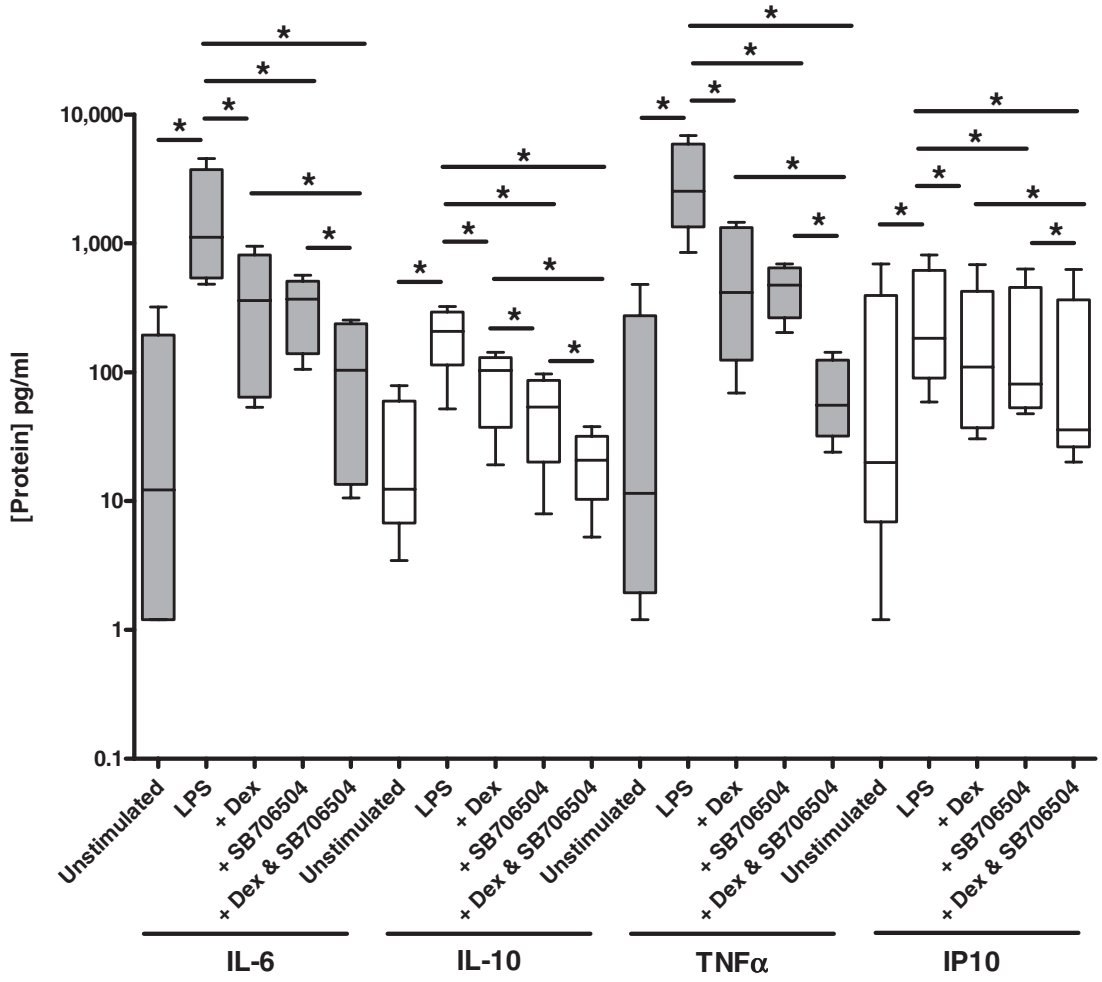

Fig. 4. Comparison of dexamethasone, SB706504, and cotreatment inhibition of cytokine levels measured by Luminex in LPS-stimulated COPD MDMs ($n = 6$). Data are presented as medians with interquartile range and range. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

all these cytokines compared with treatment with dexamethasone or SB706504 alone.

AM Cell Culture

AMs from 10 smokers with normal lung function and nine COPD patients were stimulated with LPS. SB706504 alone and dexamethasone alone significantly reduced levels of TNFα in COPD patients (median inhibition of 67.4%, $p = 0.0098$ and 77.1%, $p = 0.002$, respectively). SB706504 alone and dexamethasone alone significantly reduced TNFα production in smokers (median inhibition of 76.1%, $p = 0.001$ and 78.9%, $p = 0.002$) (Fig. 6). The effect of dexamethasone was similar in COPD patients and smokers, whereas SB706504 caused greater inhibition in smokers compared with COPD patients (76.1 versus 67.4%, $p = 0.047$). Combined therapy caused the greatest magnitude of TNFα inhibition, with a median inhibition of 91.1 ($p = 0.002$) and 91.8% ($p = 0.001$) in COPD patients and smokers, respectively.

Having observed that combined treatment with dexamethasone and SB706504 was greater than the individual components using the TLR2 and 4 agonist LPS, we then evaluated the same phenomenon using the specific TLR4 agonist UP-LPS in seven COPD patients and seven smokers (Fig. 6). Dexamethasone alone and SB706504 alone caused similar reductions in TNFα production from COPD patients (median inhibition of 88.0%, $p = 0.008$ and 83.7%, $p = 0.008$, respectively) and smokers (median inhibition of 86.1%, $p = 0.008$ and 76.9%, $p = 0.008$). Near maximal inhibition of cytokine

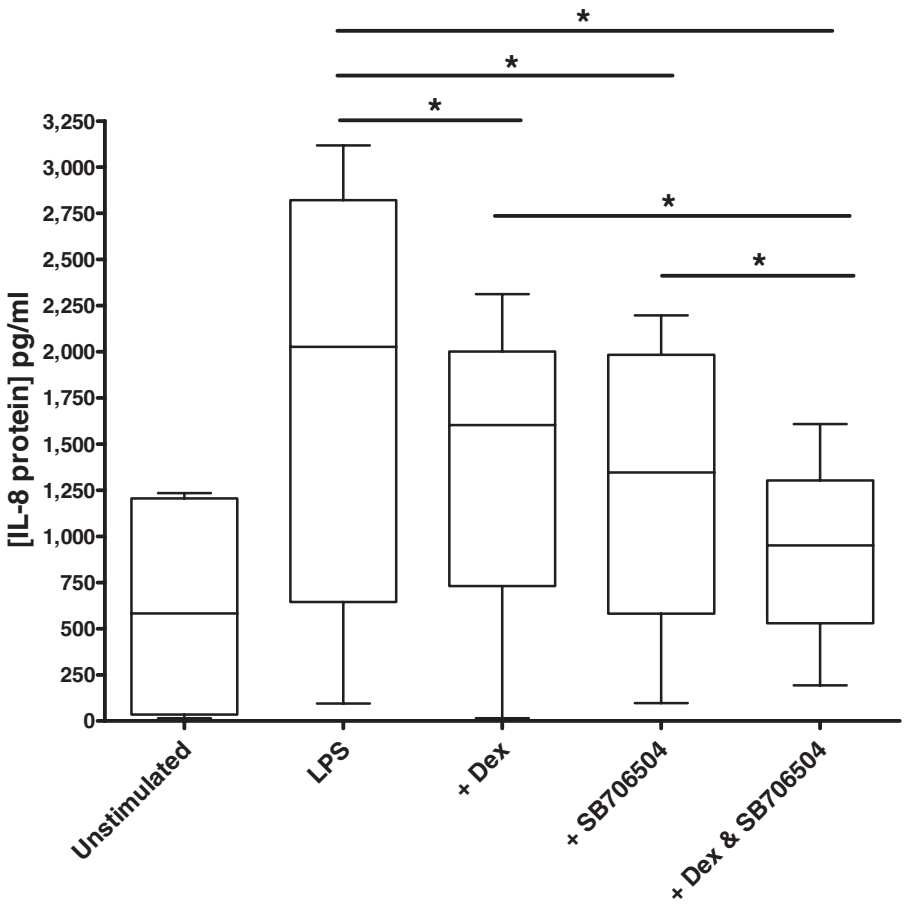

Fig. 5. Comparison of dexamethasone, SB706504, and cotreatment inhibition of IL-8 protein levels measured by ELISA in unstimulated and LPS-stimulated COPD MDMs ($n = 6$). Data are presented as medians with interquartile range and range. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

production was achieved when both drugs were used together; the median inhibition was 94.6% in COPD patients ($p = 0.008$) and 95.6% in smokers ($p = 0.008$). There were no differences between groups for the effects of these drugs ($p > 0.05$ for all comparisons).

Discussion

SB706504 significantly inhibited the transcription of a range of inflammatory genes in COPD MDMs. Combination treatment with SB706504 and dexamethasone in AMs and MDMs maximized the suppression of cytokine production, underscoring the possibility that this may be an effective anti-inflammatory strategy in COPD patients. However, 27 inflammatory genes activated by LPS in COPD MDMs were insensitive to the effects of dexamethasone. Furthermore, 23 of these genes were insensitive to dexamethasone and SB706504, suggestive of a subset of "drug-resistant" inflammatory genes in COPD macrophages.

The numerical mean of the fold change suppression of gene expression caused by SB706504 alone (−2.04) and dexamethasone alone (−3.39) was less than the fold change suppression when these two drugs were administered together (−8.90). This is suggestive of a synergistic interaction, such as GC up-regulation of mitogen-activated protein kinase phosphatase (Lasa et al., 2002) or p38 MAPK inhibition of GR (Irusen et al., 2002; Szatmáry et al., 2004). Further studies using COPD macrophages are needed to address these potential mechanisms.

We identified four genes by microarray analysis that were GC-resistant, occupying the same locus on chromosome 4 (q12-q13); CXCL1 (GROα), CXCL2 (GROβ), CXCL3 (GROγ), and IL-8. In addition, for three of these genes, there was also no significant inhibition when SB706504 was used but significant inhibition when both drugs were used. This suggests a synergistic interaction between these drugs or "unlocking" of GC insensitivity by p38 MAPK inhibition. The close proximity of these genes on a single chromosome raises the possibility of chromatin remodeling at this locus. Saccani et al. (2002) have shown previously that LPS stimulation of dendritic cells results in p38-dependent histone 3 phosphorylation at the promoter regions of IL-6, IL-8, 12p40, and monocyte chemotactic protein (CCL2), although not at TNFα and macrophage inflammatory protein (CCL3). Such phosphorylation results in increased transcriptional potential because of unwinding of chromatin. Similarly, in the current study, it is possible that SB706504 inhibited LPS-induced changes to chromatin structure, which may have allowed increased GC effects. In COPD alveolar macrophages, decreased histone deacetylase activity caused by oxidative stress is thought to lead to chromatin remodeling associated with decreased GC sensitivity (Cosio et al., 2004). Our study deals with TLR stimulation rather than oxidative stress but suggests that the four chemokines located at chromosome 4 have reduced GC sensitivity, and studies of the chromatin structure of this gene in COPD macrophages are warranted.

There are previous data from COPD AMs that IL-8 is GC-resistant (Culpitt et al., 2003; Cosio et al., 2004). These previous studies add further weight to our findings that macrophage IL-8 production is GC insensitive. This probably

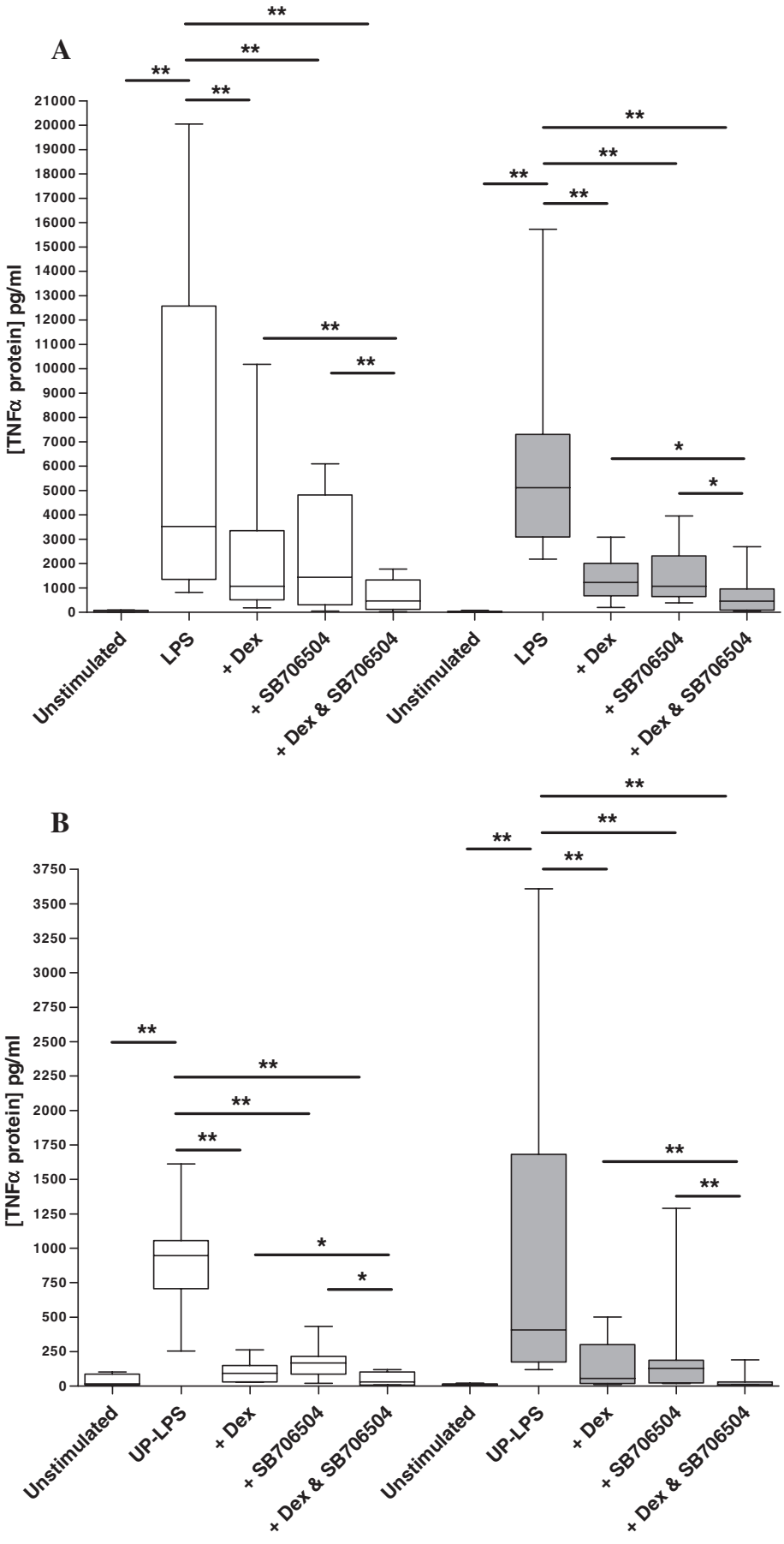

Fig. 6. Comparison of dexamethasone, SB706504, and cotreatment inhibition of TNFα protein measured by ELISA in LPS-stimulated COPD (clear bars, $n = 9$) and HS (gray bars, $n = 10$) (A) and UP-LPS-stimulated COPD (clear bars, $n = 7$) and HS (gray bars, $n = 7$) AMs (B). Data are presented as medians with IQR and range. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

has considerable therapeutic implications because IL-8 appears to play a key role in COPD pathophysiology; the levels of this neutrophil chemoattractant are raised in the lungs of COPD patients (Keatings et al., 1996) and are associated with the rate of disease progression (Wilkinson et al., 2003). Likewise, there are previous data that macrophage production of IL-8 is less sensitive to the effects of p38 inhibitors compared with other cytokines (Smith et al., 2006; Tudhope et al., 2008). We also observed this phenomenon.

The chemokines CXCL1, 2, and 3 constitute the three members of the GRO family, GROα, GROβ, and GROγ, respectively. CXCL1 expression is increased in lung tissue (Tomaki et al., 2007) and induced sputum (Traves et al., 2002) from COPD patients compared with controls. GRO family members cause neutrophil chemotaxis; therefore, our findings that macrophage-derived GRO production is GC insensitive may have implications in disease pathophysiology.

We identified 23 inflammatory genes that were up-regulated by LPS in MDMs but were insensitive to any of the treatments used. These included the cytokines IL-1β, IL-15, and IL-18 and the chemokines CCL1, 3, 4, 5, 18, 20, and 23 and CXCL5, 11, and 13. IL-1β and IL-18 share sequence homology and are members of the IL-1 family. IL-1β gene expression is raised in COPD lung tissue (Tomaki et al., 2007), whereas there is also evidence for IL-18 overexpression in COPD (Imaoka et al., 2008). Other GC-insensitive inflammatory genes implicated in the pathogenesis of COPD include CXCL11 (Costa et al., 2008), CCL5 (Costa et al., 2008), CCL3 (Di Stefano et al., 1998), and CCL20 (Demedts et al., 2007) in the airways of COPD patients.

We decided a priori to assess IL-1β, IL-6, GM-CSF, TNFα, and IL-8 mRNA levels by qPCR. Using a criterion of $p < 0.05$ in the qPCR analysis, we found that all of these genes were significantly suppressed by both drugs but that IL-1β and IL-8 were the least sensitive genes. This confirmed the array analysis where IL-1β and IL-8 were both GC-resistant. It should be noted that we used the criterion of $p < 0.01$ and a fold change of >2 to define a significant change, as is common practice in array studies.

IPA analysis was used to generate a network of the possible relationships between GC-insensitive genes, and the same approach was used for p38 MAPK inhibitor-insensitive genes. This is an accepted approach for creating hypothetical gene networks utilizing known mechanistic relationships between proteins and complexes (Juric et al., 2007; Li et al., 2007; Savli et al., 2008). The IPA network allows genes that are "highly connected" and, therefore, more likely to be involved in inflammatory processes to be identified. This approach is "hypothesis generating," and we observed that IL-1β, IL-8, IL-18, and CCL5 were centrally involved in GC resistance. Because the levels of all of these inflammatory mediators are raised in COPD patients (Keatings et al., 1996; Tomaki et al., 2007; Costa et al., 2008; Imaoka et al., 2008), then these cytokines and chemokines could be considered key culprits in GC-resistant inflammation in COPD patients.

IL-1β, IL-8, and IL-18 were also centrally involved in resistance to the effects of SB706504. These genes may be regulated by the same transcription factor, probably NFκB as suggested by both IPA networks. It should be stressed that these networks do not prove this mechanism but provide the hypothesis that IL-1β, IL-8, and IL-18 are regulated by NFκB signaling, which is both GC and p38 MAPK inhibitor-resistant in COPD macrophages.

Our gene array study used MDMs as a surrogate for AMs. There are similarities between MDMs and AMs for studying the pharmacological effects of p38 MAPK inhibitors (Tudhope et al., 2008). In accordance, we postulate that the GC and p38 MAPK inhibitor-resistant inflammatory genes that we observed in MDMs are also likely also to be resistant to the effects of these drugs in AMs. We used a group of severe COPD patients for the gene array study. It would certainly be of interest to study whether the same findings were true in milder COPD patients and controls to study whether any of our findings are dependent on the presence or severity of COPD.

p38 MAPK inhibitors may act at the level of transcription, post-transcription, translation, or post-translationally (Wang et al., 1999; Winzen et al., 1999; Brook et al., 2006). In MDMs from COPD patients, gene arrays and qPCR showed that SB706504 inhibited the transcription of a range of inflammatory genes. SB706504 was less effective than dexamethasone at inhibiting transcript abundance, particularly evident in the qPCR data. However, the effects of SB706504 and dexamethasone alone on protein levels were generally similar. This suggests that SB706504 has significant post-transcriptional effects that influence the levels of secreted inflammatory proteins. Likewise, Birrell et al. (2006) reported that the inhibitory effects of SB239063 and 2,1(1,3-dihydroxyprop-2-yl)-4-(4-fluorophenyl)-5-(2-phenoxypyrimidin-4-yl)imidazole on AM IL-6 production were predominantly at the level of protein production rather than mRNA expression (Birrell et al., 2006). The effects of p38 MAPK inhibitors on transcription may be concentration dependent, which is worthy of further exploration.

LPS-induced TNFα production was significantly suppressed by SB706504 in COPD patients and smokers, although inhibition was marginally greater in smokers compared with COPD patients (76.1 and 67.4%, $p = 0.047$). SB706504 was also found to significantly inhibit TNFα production when stimulated by the specific TLR4 agonist UP-LPS, with similar inhibition in COPD patients compared with smokers. The sample size used was not specifically statistically powered to enable a sensitive comparison of drug effects between COPD patients and smokers. COPD AMs are known to be less GC sensitive than those from controls (Culpitt et al., 2003); perhaps a larger study may elucidate any such difference when p38 MAPK inhibitors are used.

In summary, we have shown that the novel p38 MAPK inhibitor SB706504 has significant inhibitory effects on inflammatory mediator production from COPD macrophages. Importantly, we provide evidence that using this p38 MAPK inhibitor with GC can provide enhanced anti-inflammatory effects. However, we also demonstrate a subset of macrophage inflammatory genes that are resistant to the effects of these anti-inflammatory drugs, and some of these are known to be involved in the pathophysiology of COPD. Targeting these genes with different anti-inflammatory interventions may be needed.

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